Published in final edited form as:

J Clin Immunol. 2010 July ; 30(4): 502–506. doi:10.1007/s10875-010-9386-5.

Innate immune detection of bacterial virulence factors via the NLRC4 inflammasome

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Abstract

Cytokine production by innate immune cells is initiated by signaling downstream of pattern recognition receptors, including Toll-like receptors. A subset of cytokines, including IL-1b and IL-18, require post-translational proteolysis before secretion, which provides a second mechanism of regulation. This proteolysis is dependent upon caspase 1, which is activated by Nod-like receptor (NLR) signaling. NLRC4 (previously named Ipaf) activates caspase 1 in response to bacterial virulence factors including type III and IV secretion systems (T3SS and T4SS). NLRC4 recognizes T3SS/T4SS in two ways: indirectly by detecting flagellin, and directly by detecting the T3SS rod protein. Both flagellin and rod protein are unintentionally delivered to the mammalian cytosol by the bacterium through the T3SS.

Keywords

NLRC4; Caspase 1; IL-1 β ; type III secretion; inflammation

Introduction

Macrophages in the innate immune system direct inflammatory responses to infection. They reside within tissues where they act as sentinels that detect the presence of microorganisms and secrete cytokines and chemokines that recruit other immune cells to the site of the infection. Macrophages use pattern recognition receptors (PRRs) to detect microbe associated molecular patterns (MAMPs), which are conserved among microorganisms but which are not present in host tissue. Many MAMPs are detected via Toll-like receptors (TLRs), and a large array of inflammatory mediators are synthesized and secreted in response.

Inflammation, mediated by cytokine expression, is a tightly regulated process. Excessive inflammation results in tissue damage whereas insufficient inflammation will fail to control the infection. However, determining the appropriate magnitude of inflammation requires more sophistication than simply detecting the concentration of MAMPs in the tissue. Similar microorganisms may have similar levels of MAMPs but possess vastly divergent potentials for causing disease based on the virulence determinants they express. For example, two closely related gram negative bacteria, *Salmonella typhimurium* and a commensal *Escherichia coli* have similar LPS content, but *S. typhimurium* expresses a variety of virulence factors (1). This makes *S. typhimurium* more likely to disseminate and cause systemic infection than commensal *E. coli*. Further, different *E. coli* strains vary in their

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expression of virulence factors. Most *E. coli* found in the gut are commensal nonpathogens. Other strains encode virulence factors that will enable them to cause disease: enteropathogenic *E. coli* (EPEC) promote diarrhea and enterohemorrhagic *E. coli* (EHEC) express several additional virulence factors which can result in hemolytic uremic syndrome(2). Clearly *S. typhimurium*, EPEC and EHEC are much more dangerous to the host than very closely related avirulent commensal *E. coli*. Therefore, it is important for hosts to distinguish between virulent and avirulent microorganisms.

Two cytokines, IL-1 β and IL-18, are differentially secreted in response to pathogens and non-pathogens. IL-1 β promotes inflammation, induces febrile responses and recently has been shown to stimulate Th17 cells. IL-18 stimulates IFN- γ production from NK cells and Th1 T cells. Both are induced in response to TLR stimuli but are not secreted. IL-1 β and IL-18 are retained in the cytosol until they are cleaved to their mature form by caspase 1. Caspase 1 activity is regulated by proteins in the Nod-like receptor family (NLRs), including NLRP3 and NLRC4. Thus, IL-1 β and IL-18 secretion are regulated in a two-step fashion. First, TLRs induce their expression, and then NLRs induce their post-translational processing. This enables enhanced immune responses to pathogenic bacteria. In this review we will discuss some of the mechanisms by which IL-1 β and IL-18 secretion are regulated.

Type III and IV secretion systems

Bacterial type III and type IV secretion systems (T3SS and T4SS) are major virulence factors in most Gram negative pathogens(3). Although evolutionarily distinct, they both function to transfer effector proteins directly from the bacterial cytosol into the host cell cytosol. The T3SS apparatus forms a “needle complex” that spans the bacterial membranes and periplasm with a short needle protruding from the cell surface. The needle complex is hollow, and secreted proteins pass through the interior of the structure in an unfolded conformation. This mechanism is used by most Gram negative pathogens to promote virulence. Although the structural components and mechanism of transport are conserved between T3SS in different bacteria, the effectors delivered can be widely divergent, permitting a variety of virulence strategies. For example, the *Salmonella* SPI1 T3SS and the *Shigella* Mxi/Spa T3SS promote epithelial cell invasion, while the EPEC/EHEC LEE T3SS promotes the formation of a pedestal on the surface of epithelial cell to which the bacteria adhere. Some bacteria, including *Salmonella* spp., encode two distinct T3SS that promote different aspects of virulence and transfer different effector proteins(3). Similarly, T4SS have different effects on host cells based on the complement of effectors translocated; the *L. pneumophila* Dot/Icm T4SS promotes intracellular replication while the *Helicobacter pylori* cag PAI T4SS alters the cytoskeleton and proliferation of epithelial cells (4).

The complement of effector proteins transferred is variable between bacterial species and strains. Many single effectors can be gained or lost from pathogens with only incremental effects on overall virulence. Consequently, they are poor targets for direct detection by PRRs. Instead, mammals monitor for bacterial virulence by detecting the activity of T3SS/T4SS through both direct and indirect mechanisms.

Indirect detection of T3SS and T4SS: cytosolic flagellin detection via NLRC4

NLRC4 is the cytosolic sensor for T3SS/T4SS activity, activating caspase 1 dependent IL-1 β and IL-18 secretion in response. It has a typical NLR domain architecture, composed of an amino terminal signaling domain (in this case a CARD), a central oligomerization domain, and carboxy terminal leucine rich repeats. NLRC4 can directly recruit caspase 1 via

CARD-CARD interactions. Naip5 and ASC also play roles in NLRC4 signaling, but are not required for all responses (5).

NLRC4 detects T3SS and T4SS indirectly by taking advantage of rare errors made by bacteria in selecting proteins for T3SS mediated translocation. A small number of flagellin monomers are inadvertently translocated along with the larger quantity of effector proteins. However, flagellin is highly conserved between bacteria, so it serves as an ideal target for innate immune recognition. Detection of T3SS indirectly via flagellin transfer enables NLRC4 to detect T3SS/T4SS activity during infection with *S. typhimurium*, *L. pneumophila*, *Pseudomonas aeruginosa*, EPEC, and likely many more bacteria (5–9). The most attractive hypothesis to explain NLRC4 activation by flagellin is that the LRR domain directly binds flagellin. However, direct interaction between NLRC4 and flagellin has not been demonstrated. Whether NLRC4 directly binds flagellin, interacts with a co-receptor, or responds to some other secondary effect of flagellin on cytosolic processes remains to be determined.

Why is flagellin mistakenly translocated into the host cell cytosol by the virulence-associated T3SS? This is likely explained by the evolutionary and structural relationship between virulence-associated T3SS and the flagellar hook-basal body (HBB) export apparatus. (Note that the HBB is also classified as a “flagella-associated T3SS” but we use the nomenclature HBB in this review; we refer to the “virulence-associated T3SS” as T3SS in this review.) Normally, flagellin monomers are transferred from the bacterial cytosol through the hollow HBB apparatus for polymerization at the tip of the hook and then at the leading cap of the flagellar filament (10). Likewise, T3SS effectors are transferred through the hollow needle complex into host cells. Most proteins are targeted to the appropriate apparatus by specific chaperones, and subsequently exported in an unfolded conformation. Recognition of targets is highly specific, but not perfect. Small quantities of flagellin are inappropriately targeted for secretion via the virulence-associated T3SS (6,11) and small quantities of effectors can be inappropriately targeted to the flagellar apparatus (12). Virulence associated T4SS also are believed to transfer small amounts of flagellin into the cytosol of host cells, although the mechanism for inappropriate substrate recognition of flagellin by T4SS is less apparent.

Detection of flagellin within the cytosol serves not only as a marker for bacterial T3SS/T4SS activity, but also as a marker for the presence of flagellated bacteria within the cytosol. *Listeria monocytogenes* is a flagellated Gram positive bacteria that lyses the phagosome and escapes into the cytosol of host cells, where it replicates. Once in the cytosol, *L. monocytogenes* flagellin can be detected by NLRC4 (13).

Direct detection of T3SS: cytosolic rod protein

Pathogens can evade flagellin based NLRC4 detection by mutating or transcriptionally repressing the flagellin gene. The primary example of this are *Shigella* spp, which utilize T3SS to invade epithelial cells and are aflagellate, carrying multiple mutations within flagellar T3SS apparatus genes. Nevertheless, *Shigella flexneri* T3SS is detected via NLRC4 (14). Because the same sensor was responsible for T3SS detection, this finding seemed to challenge the hypothesis that NLRC4 detects T3SS via flagellin transfer. Additional lines of evidence indicated that *S. typhimurium* and *P. aeruginosa* T3SS were detected by both flagellin-dependent and flagellin-independent mechanisms (8,15).

How can a single sensor detect both flagellin and a second marker of T3SS? To resolve this apparent paradox, a comparison of the structure of the T3SS and HBB apparatus is required. As noted above, flagellin is exported by the HBB and assembles into a hollow tube; this hollow tube architecture is characteristic of both the HBB and virulence-associated T3SS.

The tube structure is divided into different segments that are comprised of distinct proteins, each of which polymerizes in a helical array. In the virulence-associated T3SS apparatus, the tube is formed by a two structural components: the rod and needle(16). The rod component spans the periplasmic space between the bacterial inner and outer membranes while the needle protrudes from the bacterial surface and spans the extracellular space between the bacterial outer membrane and host cell plasma membrane.

The bacterium assembles the T3SS by first secreting the basal body components through the Sec secretion apparatus, where it assembles into a hollow shell. Rod proteins are then secreted via the T3SS export complex directly into the shell, where they polymerize and form the lining of the hollow tube (Figure 1) (16). Subsequently, needle monomers are exported through the T3SS apparatus, pass through the now completed rod and polymerize at the end of the rod component. We hypothesized that, like flagellin, rod and needle proteins may be translocated into the host cell cytosol. Under this hypothesis excess rod/needle protein in the bacterial cytosol could be translocated into the host cytosol after completion of the needle complex (Figure 1). Alternately, rod protein from the interior of the assembled complex may slough into the channel and pass through the needle into host cells (Figure 1).

If the needle or rod proteins are inappropriately transferred into host cells, they could serve as a good target for innate immune detection because of their greater conservation between all T3SS than translocated effectors proteins. When we examined these two proteins, we found that the rod component from multiple bacteria was detected via NLRC4 (6). Flagellin and rod proteins, but not needle or hook proteins, share sequence conservation at their carboxy termini that is required for NLRC4 detection of both proteins. The reason for this carboxy terminal conservation between rod proteins and flagellins is currently unknown. Interestingly, the conserved residues in this motif within the flagellin monomer face the interior wall of the tube(6,17). It may be that certain biophysical properties are required to form the interior lining of the hollow tube. However, this hypothesis does not explain why the NLRC4 detected motif is found in flagellin and the virulence-associated rod, but not found needle, hook, or flagellar-associated rod proteins.

Although purified flagellin and rod protein have similar potency after transfection into macrophages, detection of the rod proteins appears to be less sensitive than detection of flagellin during bacterial infections(6). This may be explained by the relative abundance of flagellin monomers and rod monomers within the bacterial cytosol. Flagellin is one of the most highly expressed proteins in bacteria and is quite stable; in contrast, the rod is expressed in much lower abundance, and is readily degraded when not integrated into the T3SS needle complex (18,19). Thus, the available pool of flagellin is much greater than rod for mistaken translocation into host cells.

Evasion of NLRC4

The role of NLRC4 in detecting T3SS via flagellin and rod proteins was first demonstrated by investigators studying *S. typhimurium* and *L. pneumophila* interactions with macrophages (5). However, mounting evidence indicates that *S. typhimurium* evades NLRC4 *in vivo* (6,20). How then can *S. typhimurium* both be detected by and evade NLRC4? The key to explaining this apparent paradox is that NLRC4 is expressed by macrophages and not by epithelial cells, and that *S. typhimurium* use different virulence factors to target the two cell types.

S. typhimurium expresses two distinct T3SS systems, SPI1 and SPI2. While SPI1 T3SS is expressed primarily in the gastrointestinal tract and promotes bacterial invasion of epithelial cells, SPI2 is primarily expressed in the intracellular environment and promotes intracellular

replication within macrophages. SPI1 and SPI2 T3SS utilize distinct apparatus and effector genes including the rod component: PrgJ for SPI1 and SsaI for SPI2. Interestingly, while PrgJ is detected by NLRC4, SsaI is not. Further, flagellin is expressed in the gastrointestinal tract concomitantly with SPI1, and is repressed in the vacuole when SPI2 is expressed. Therefore, when in the intracellular environment, *S. typhimurium* evade NLRC4 by repressing flagellin and expressing a rod protein that is not detected by NLRC4. When *S. typhimurium* are altered to express a rod protein that is detected by NLRC4, they are efficiently cleared during systemic infection, highlighting the importance of NLRC4 evasion (6). This indicates that efficient activation of NLRC4 would render *S. typhimurium* completely avirulent.

However, *S. typhimurium* are not able to completely evade NLRC4 during infection. Caspase 1 deficient mice have an increased susceptibility to *S. typhimurium* infection (20,21) and this has recently been attributed to a combined effect of NLRC4 and NLRP3 signaling (P. Broz and D. Monack, personal communication). Again, there is an apparent discrepancy, this time between the observation of NLRC4 evasion mechanisms and data indicating that NLRC4 contributes to *S. typhimurium* resistance in mice. This could also be explained by differential expression of flagellin, SPI1 and SPI2 during infection *in vivo*. It is possible that as the bacteria pass the epithelial barrier in the gastrointestinal tract they may express residual SPI1 and/or flagellin as they encounter macrophages for the first time. In this window, the innate immune system would have the opportunity to respond to PrgJ rod protein or flagellin via NLRC4. However, at later times, after the bacteria have successfully replicated within macrophages, the repression of SPI1 and flagellin may be more complete, and *S. typhimurium* will subsequently evade NLRC4 during the systemic phase of infection. Therefore, it may be at this transition from the gastrointestinal phase to the systemic phase where NLRC4 detection may permit increased bacterial control. The innate immune system may be capitalizing on a short window where proper expression of genes for systemic virulence has not yet been achieved by the bacteria.

Concluding remarks

The innate immune system responds to markers of virulence in addition to markers of microbes. The term pathogen associated molecular patterns (PAMPs) was first associated with structures such as LPS, but the term MAMPs is gaining wider acceptance to denote that these structures are conserved amongst both pathogens and non-pathogens. It is important for the innate immune system to recognize all microorganisms present in sterile tissues; however it is equally important to differentiate pathogens from non pathogens. In contrast to MAMPs, certain molecular patterns are characteristic of pathogens and not present in nonpathogens; these may be considered as virulence associated molecular patterns (VAMPs). VAMPs thus permit the immune system to differentiate microbes based on their potential to cause disease. Interestingly, flagellin can be considered both a MAMP and a VAMP. When present in the extracellular space, flagellin is a MAMP detected by TLR5 and will not differentiate between pathogenic and non-pathogenic bacteria. However, only pathogens will introduce flagellin into the cytosol, either by vacuolar lysis or T3SS/T4SS mediated delivery; thus cytosolic flagellin is a VAMP. T3SS rod proteins, on the other hand, are always characteristic of pathogens, and thus would always be considered VAMPs. Further investigation into host cells detection of MAMPs and VAMPs and the mechanisms by which microbes evade detection will enhance our understanding of the fundamental nature of the evolutionary arms race between pathogens and immune system.

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Figure 1. Model for assembly of the rod component and its translocation into host cells

A model reflecting current understanding of T3SS rod component assembly is shown. The hollow exterior shell of the basal body is first assembled, spanning the bacterial inner membrane (IM), periplasm and outer membrane (OM). Rod monomers (red) are secreted by the T3SS into the interior of the basal body shell, where they polymerize to form the rod. This process is repeated for the needle that spans the extracellular space between the bacterial surface and the host cell plasma membrane (PM). Rod monomers are proposed to be translocated from excess monomers in the bacterial cytosol, or from polymerized rod proteins that slough into the internal channel of the T3SS needle complex. The evolutionarily conserved flagellar apparatus is shown at right; excess flagellin monomers in the bacterial cytosol are believed to be transported through the T3SS into the host cell cytosol.